METHODS

Cloning of BM4

BM4 was generated by PCR amplification of mutated fragments of *bet v 1.0101* (X15877). The mutated fragments were created by using internal mismatch primers (Table E1). After PCR amplification, the DNA fragments were gel-purified (Wizard SV Gel and PCR Clean Up system; Promega, Madison, Wis), pooled, and assembled in a primerless PCR. Full-length cDNAs were amplified by using the primer pair BetF and BetR (Table E1).

Expression and purification of BM4

Freshly transformed *E coli* BL21 (DE3; Invitrogen Corp, Carlsbad, Calif) cells were grown at 37°C to an OD600 of 0.8. Protein expression was induced by addition of 0.5 mmol/L isopropyl-β-d-thiogalactopyranoside (IPTG). After incubation for 6 hours, cells were harvested and resuspended in 50 mmol/L TrisBase, 1 mmol/L EDTA, and 0.1% Triton X-100. Cells were lysed, cellular debris and insoluble proteins were precipitated, and thereafter the pellet was washed once with 50 mmol/L TrisBase, 1 mmol/L EDTA, and 1% Triton X-100, and once with 25% ethanol, 5 mmol/L sodium phosphate pH 7.4. After centrifugation for 30 minutes at 25,000*g*, the pellet was dissolved in 6 mol/L urea, 20 mmol/L sodium phosphate pH 7.4, and centrifuged, and this supernatant was applied on a Q-Sepharose column (GE Healthcare, Little Chalfont, United Kingdom). BM4 remained in the flow-through. After addition of 2.2 mol/L NaCl, BM4 was purified by using a Phenyl-Sepharose column followed by a polishing step using a SP-Sepharose column (GE Healthcare). Fractions containing BM4 were pooled and dialyzed against 6 mol/L urea, 20 mmol/L sodium phosphate pH 7.4, followed by dialysis against 5 mol/L urea, 10 mmol/L sodium acetate pH 5. A final polishing step was performed by using a Q-Sepharose column (GE Healthcare). Recombinant BM4 was dialyzed against 5 mmol/L sodium phosphate buffer pH 7.4 and stored at –20°C.

ANS binding

Ligand binding to Bet v 1 and its derivatives was analyzed by fluorescence spectroscopy using deoxycholate as substrate and ANS as a competitor. Assay solutions consisted of 10 μmol/L of the respective allergen, either with or without 100 μmol/L deoxycholate, and 50 μmol/L ANS in 5 mmol/L sodium phosphate buffer pH 7.4. Measurements were performed from 410 to 600 nm in a Tecan Infinite 200 microplate reader (Tecan Group Ltd, Männedorf, Switzerland) by using an excitation wavelength of 370 nm.[E1]

ELISA experiments

For ELISA experiments, Maxisorp plates (Nalge Nunc, Rochester, NY) were coated with allergen (200 ng/well in 50 μL PBS) overnight at 4°C. Plates were blocked with TBS, pH 7.4, 0.05% (vol/vol) Tween, 1% (wt/vol) BSA, and incubated with patients' sera diluted 1:10 overnight at 4°C. Bound IgE was detected with alkaline phosphatase-conjugated monoclonal antihuman IgE antibodies (BD Biosciences, Franklin Lakes, NJ) after incubation for 1 hour at 37°C and 1 hour at 4°C. 4-Nitrophenyl phosphate 10 mmol/L (Sigma-Aldrich, St Louis, Mo) was used as substrate, and OD was measured at 405/492 nm. All measurements were performed as triplicates; results are presented as mean OD values. For inhibition assay using murine immune sera, the murine sera were preincubated for 2 hours at 56°C to deplete serum IgE, and coated antigen was incubated with murine sera (dilution 1:20) for 16 hours at 4°C. Human sera were added in a 1:10 dilution, and bound IgE was detected as described.

Recombinant production of Bet v 1*

A batch of Bet v 1.0101 was produced in the laboratory and is labeled Bet v 1* throughout the manuscript to distinguish it from commercially bought Bet v 1. *Bet v 1.0101* (X15877) was cloned into a pET28b vector (Novagen; Merck KGaA, Darmstadt, Germany) using NcoI and EcoRI restriction sites. Freshly transformed *E coli* BL21 (DE3; Invitrogen Corp) cells were grown at 37°C to an OD600 of 0.8. Protein expression was induced by addition of 0.5 mmol/L IPTG. After incubation for 6 hours, cells were harvested and resuspended in 25 mmol/L imidazole pH 7.4, 0.1% Triton X-100. Cells were lysed, cellular debris and insoluble proteins were precipitated, and thereafter NaCl was added to a final concentration of 1 mol/L. After lowering the pH to 4.8 by the addition of NaH_2PO_4, the solution was centrifuged for 30 minutes at 25,000*g*, and the supernatant was applied on a Phenyl-Sepharose column (GE Healthcare). Bet v 1* was purified by gradient reduction of NaCl. Fractions containing Bet v 1* were pooled and dialyzed against 25 mmol/L imidazole pH 7.4. A final polishing step was performed by using a Q-Sepharose column (GE Healthcare). Recombinant Bet v 1* was dialyzed against 5 mmol/L sodium phosphate buffer pH 7.4 and stored at –20°C. Endotoxin content was 1.31 ng/mg as determined by LAL assay (Associates of Cape Cod, Inc, East Falmouth, Mass).

Recombinant production of A1-6

The Bet v 1.0101 mutant allergen A1-6,[E2] which has amino acid exchanges at 6 positions, was cloned into a pET28b vector (Novagen; Merck KGaA) by using NcoI and EcoRI restriction sites. Freshly transformed *E coli* BL21 (DE3; Invitrogen Corp) cells were grown at 37°C to an OD600 of 0.8. Protein expression was induced by addition of 0.5 mmol/L IPTG. After incubation for 18 hours at 16°C, cells were harvested and resuspended in 25 mmol/L sodium phosphate buffer pH 7.4, 0.5 mol/L urea. Cells were lysed, cellular debris and insoluble proteins were precipitated, and thereafter NaCl was added to a final concentration of 1 mol/L. After lowering the pH to 4.8 by the addition of NaH_2PO_4, the solution was centrifuged for 30 minutes at 25,000*g*, and the supernatant was applied on a Phenyl-Sepharose column (GE Healthcare), followed by a gradient reduction of NaCl to elute A1-6. Fractions containing the desired protein were pooled and dialyzed against 25 mmol/L imidazole pH 7.4. A final polishing step was performed by using a Q-Sepharose column (GE Healthcare). Recombinant A1-6 was dialyzed against 5 mmol/L sodium phosphate buffer pH 7.4 and stored at –20°C. Endotoxin content was 1.34 ng/mg protein as determined by LAL assay (Associates of Cape Cod).

Physicochemical analysis of recombinant proteins

Purity of recombinant proteins was determined by SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue R-250 (Biorad, Hercules, Calif). Protein identity was determined by amino acid analysis of allergens using the Pico Tag method (Waters Corp, Milford, Mass) and by mass spectrometry using a Quadrupole time-of-flight mass spectrometer with electrospray ionization (ESI-QTOF-MS; Waters Corp).[E3] Circular dichroism spectra to determine protein secondary structure were recorded with a JASCO J-815 spectropolarimeter fitted with a PTC-423S Peltier-type single-position cell holder in appropriate buffers (Jasco, Tokyo, Japan). Homogeneity and aggregation behavior of proteins were assessed by online-size exclusion chromatography coupled with a UV detector and a triple detection array (TDA 302; Viscotek Corp, Houston, Tex) monitoring refractive index, right-angle light scattering, and intrinsic viscosity. Size exclusion chromatography triple detection array runs were performed by using a 7.8 × 300 mm TSKgel G2000SWXL column (Tosoh Bioscience, Stuttgart, Germany) on a HP1100 analytical chromatography system (Hewlett-Packard, San Jose, Calif) at 0.5 mL/min in PBS. By using the combination of data obtained by sequential UV, refractive index, intrinsic viscosity, and right-angle light-scattering detection, the approximate hydrodynamic radius of eluted proteins or aggregates was determined. In addition, DLS was performed on a DLS802 system (Viscotek Corp) at 1.0 mg/mL in 5 mmol/L sodium phosphate pH 7.4 to determine the hydrodynamic radius and polydispersity of proteins in solution.[E4]

FTIR

For FTIR spectra, data were accumulated in the temperature-controlled AquaSpec flow-through transmission cell with 7 μm path length at 25°C with a spectral resolution of 4 cm^{-1} and a data acquisition time of 100 seconds (Bruker Optics, Ettlingen, Germany). Data procession and evaluation was performed with the Opus software (Bruker Optics). Second derivatives were calculated from the absorbance spectra and vector-normalized on the amide I band. Secondary structure prediction was performed applying partial least-squares calibrations for α-helix and β-sheets. These calibrations are based on infrared transmission spectra of aqueous solutions of 43 different proteins with secondary structure reference information from x-ray crystallography.

REFERENCES

E1. Vaz DC, Rodrigues JR, Sebald W, Dobson CM, Brito RM. Enthalpic and entropic contributions mediate the role of disulfide bonds on the conformational stability of interleukin-4. Protein Sci 2006;15:33-44.

E2. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 1998;12:231-42.

E3. van Ree R, Chapman MD, Ferreira F, Vieths S, Bryan D, Cromwell O, et al. The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. Allergy 2008;63:310-26.

E4. Himly M, Nony E, Chabre H, Van Overtvelt L, Neubauer A, van Ree R, et al. Standardization of allergen products, 1: detailed characterization of GMP-produced recombinant Bet v 1.0101 as biological reference preparation. Allergy 2009;64:1038-45.

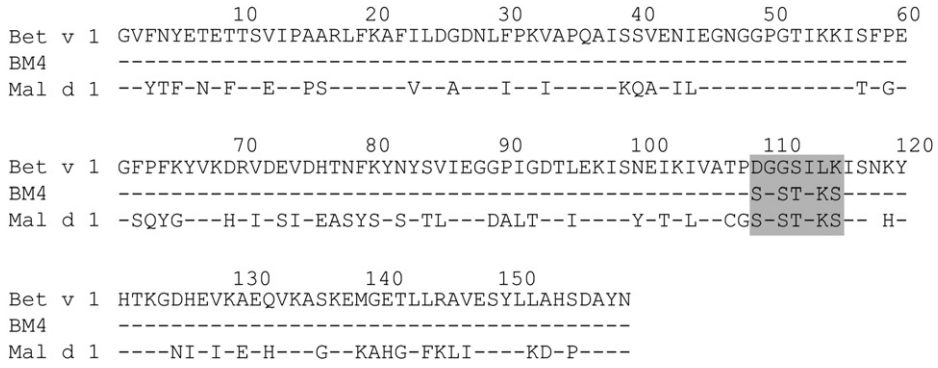

FIG E1. Primary sequence of BM4 compared to Bet v 1, isoform 0101, and Mal d 1, isoform 0108. The exchange sequence of BM4 is highlighted by a *gray box*.

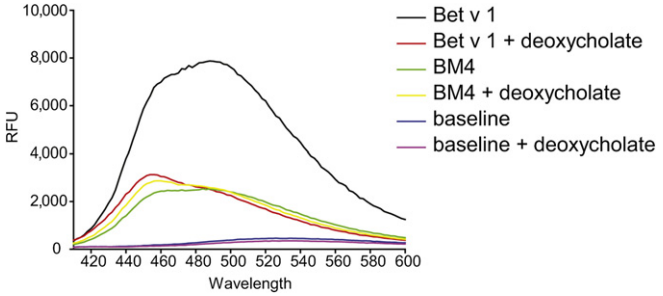

FIG E2. ANS binding to Bet v 1 and BM4 was analyzed with fluorescence spectroscopy. Data are presented in mean fluorescence units *(RFU)*.

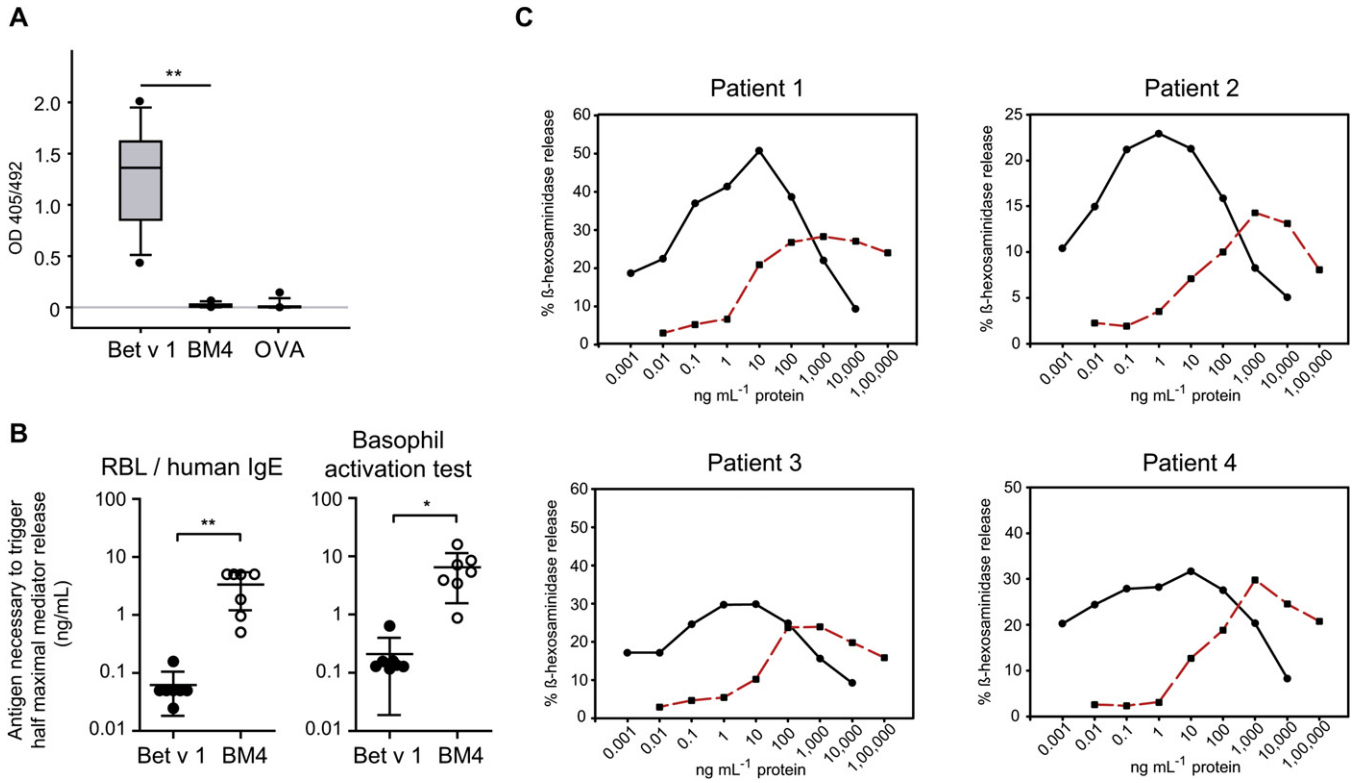

FIG E3. IgE binding of BM4 was compared with Bet v 1 by ELISA detecting antigen-specific human serum IgE (n = 13; **A**), RBL assays using patients' sera (n = 8; **B**), or basophil activation tests (n = 8; **B**). **C,** Four characteristic RBL titration curves are shown. Bet v 1 is represented as a *black line* and BM4 as a *red line* in the graphs. *OVA,* Ovalbumin.

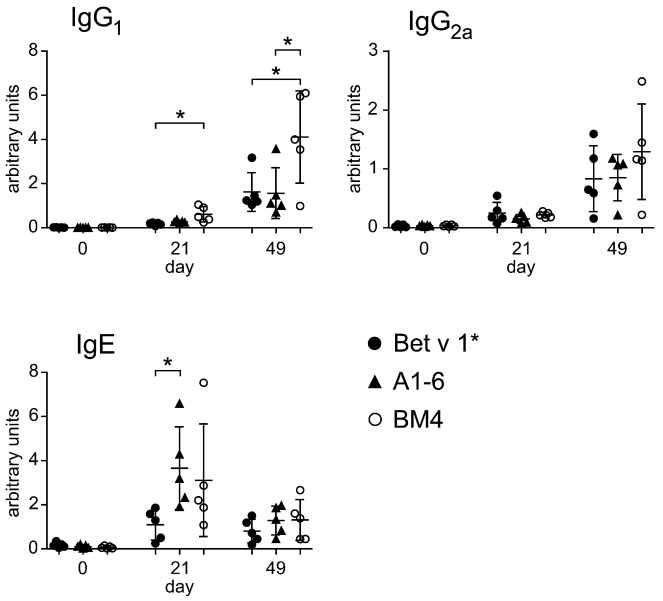

FIG E4. BALB/c mice were immunized 4 times with Bet v 1* *(filled circle)*, A1-6 *(filled triangle)*, or BM4 (*open circle*; n = 5 per group). Bet v 1*–specific antibody levels were determined by ELISA. *P* values were calculated with *t* tests (*$P < .05$).

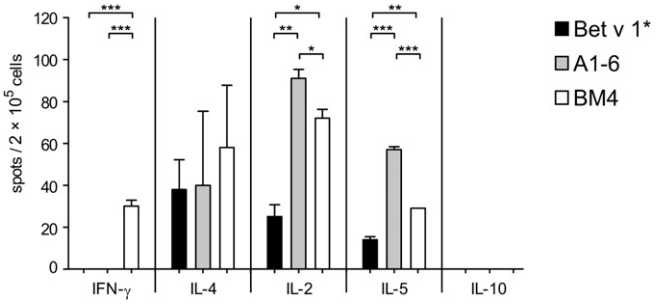

FIG E5. BALB/c mice were immunized 4 times with Bet v 1* *(black bars)*, A1-6 *(gray bars)*, or BM4 (*white bars;* n = 5 per group). ELISPOT analysis of splenocytes from immunized animals expressed as mean cytokine-secreting cells per 2×10^5 spleen cells ± SEM. *P* values were calculated with *t* tests (**P* < .05; ***P* < .01; ****P* < .001).

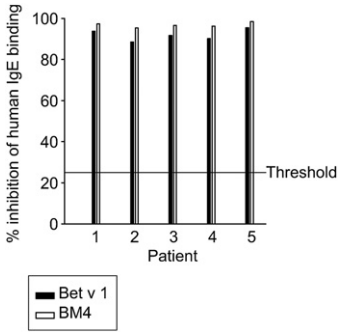

FIG E6. Bet v 1–coated ELISA plates were preincubated with IgE-depleted pooled mouse sera of animals immunized with Bet v 1 (n = 5) or BM4 (n = 5), and human serum IgE binding of 5 patients with birch pollen allergy was determined. The threshold was calculated by preincubation of Bet v 1 with pooled murine preimmune sera (n = 5).

TABLE E1. Primer list

Primer	Sequence
BM4 F	5′GCAACCCCTagTGGAaGcaCCATCaaGAgtATCAGCAAC3′
BM4 R	5′GTTGCTGATacTCttGATGGtgCtTCCActAGGGGTTGC 3′
BetF	5′CATGCCATGGGTGTTTTCAATTACGAA3′
BetR	5′GGAATTCTTAGTTGTAGGCATCGGAGTG3′

Primers used for construction of Bet v 1.0101–Mal d 1.0108 chimeric protein BM4. Exchanged bases are shown in *lower-case letters*; restriction sites are *underlined*.